ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 263, No. 1, May 15, pp. 77-85, 1988

Purification and Characterization of Cutinase from a Fluorescent *Pseudomonas putida* Bacterial Strain Isolated from Phyllosphere[1]

JOSEPH SEBASTIAN AND P. E. KOLATTUKUDY[2]

Ohio State Biotechnology Center, Rightmire Hall, The Ohio State University, Columbus, Ohio 43210-1002 and Institute of Biological Chemistry and Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99163

Received December 21, 1987

Cutinase, an extracellular enzyme, was induced by cutin in a fluorescent *Pseudomonas putida* strain that was found to be cohabiting with an apparently nitrogen-fixing *Corynebacterium*. This enzyme was purified from the culture fluid by acetone precipitation followed by chromatography on DEAE-cellulose, QAE-Sephadex, Sepharose 6B, and Sephadex G-100. The purified enzyme showed a single band when subjected to polyacrylamide electrophoresis and the enzymatic activity coincided with the protein band. Sodium dodecyl sulfate–polyacrylamide electrophoresis showed a single band at a molecular weight of 30,000 and gel filtration of the native enzyme through a calibrated Sephadex G-100 column indicated a molecular weight of 30,000, showing that the enzyme is a monomer. The amino acid composition of bacterial cutinase is distinctly different from that of fungal or plant cutinases. This bacterial cutinase showed a broad pH optimum between 8.5 and 10.5 with ^{3}H-labeled apple cutin as the substrate. Linear rates of cutin hydrolysis were measured up to 20 min of incubation time and 4 mg/ml of cutin gave the maximum hydrolysis rate. This cutinase catalyzed hydrolysis of *p*-nitrophenyl esters of C_4 to C_{16} fatty acids with decreasing V and increasing K_m for the longer chain esters. It did not hydrolyze tripalmitoyl glycerol or trioleyl glycerol, indicating that this is not a general lipase. Active serine-directed reagents such as organophosphates and organoboronic acids severely inhibited the enzyme, suggesting that bacterial cutinase is an "active serine" enzyme. Neither thiol-directed reagents nor metal ion chelators had any effect on this enzyme. Antibody raised against purified enzyme gave a single precipitin line on Ouchterlony double diffusion analysis. Western blot analysis of the extracellular fluid of induced *Ps. putida* showed a single band at 30 kDa. No immunological cross-reactivity was detected between the present bacterial enzyme and the fungal enzyme from *Fusarium solani pisi* when rabbit antibodies against either enzyme was used.

Phyllospheric bacteria have been implicated as being providers of fixed nitrogen to plants (1). Some such nitrogen fixing organisms have been isolated from the leaf surface (2-4). For example, bacteria isolated from *Phaseolus mungo* have been shown to replace nitrogenous fertilizers for wheat (4). To test whether these phyllospheric bacteria utilize plant cuticular components while living on a plant surface, some of the isolates obtained from plant surfaces were tested for their ability to grow on a nitrogen-free medium with cutin as the sole source of carbon. Isolates which could grow under such conditions

[1] This work was supported by NSF Grant DMB-8306835.

[2] To whom correspondence should be addressed at: Ohio State Biotechnology Center, Rightmire Hall, The Ohio State University, Columbus, OH 43210-1002.

0003-9861/88 $3.00

were found to produce cutinase when grown with cutin as the sole source of carbon (5). Thus, a *Pseudomonas putida* strain isolated from *Ph. mungo* was found to produce cutinase upon induction by cutin (5). Although fungal and higher plant cutinases have been purified (6), little is known about bacterial cutinase. In this paper, we report purification and properties of cutinase isolated from the *Ps. putida* isolated from the phyllosphere. This bacterial enzyme appears to be distinctly different in molecular properties from fungal and plant cutinases but the catalytic mechanism appears to be quite similar to that of the fungal enzyme.

MATERIALS AND METHODS

Golden delicious apple cutin and ^{3}H-labeled apple cutin were prepared as described earlier (7). Sepharose 6B, Sephadex G-100, QAE-Sephadex, and *p*-nitrophenyl esters with different acyl moieties were from Sigma Chemical Co. DEAE-52 cellulose was purchased from Whatman, Inc. Alkyl boronic acids were synthesized by published procedures (8). A low-molecular-weight standard protein mixture was purchased from Bio-Rad Laboratories, Richmond, California.

Purification of cutinase. Cutinase-producing fluorescent *Ps. putida* strain isolated from *Ph. mungo* (4) was maintained on nutrient broth-yeast extract (NBY) agar plates at 4°C. Inoculum for the batch preparation was made in 30 ml NBY medium supplemented with 0.4% apple cutin. Fernbach flasks containing 1 liter of the same medium were inoculated with 30 ml cultures grown for 24 h at 30°C. After 4 days of growth in shaker incubators at 30°C, the culture filtrates were collected by centrifugation at 10,000*g* and the supernatant was lyophilized. Typically, the residue from 6 liters of culture fluid was dissolved in 450 ml of 20 mM Tris-HCl buffer, pH 8; the residual insoluble material was removed by centrifugation at 10,000*g*; and the dark viscous solution was dialyzed overnight against 8 liters of the same buffer. To this enzyme solution, 3 vol of cold (−20°C) acetone were added; the mixture was stirred for 1 h in an ice bath; and the precipitated protein was collected by centrifugation at 10,000*g*.

The protein obtained by acetone precipitation was dissolved in 100 ml of 20 mM Tris-HCl buffer, pH 8, and dialyzed overnight against the same buffer. This enzyme preparation was mixed with DEAE-cellulose (150 g) that was swollen in 20 mM Tris-HCl buffer, pH 8, and equilibrated with the same buffer. After leaving the mixture for one hour, DEAE-cellulose was removed by centrifugation for 5 min at 5000*g*. The supernatant was collected; the residue was washed three times with 200 ml of the same buffer each time and centrifuged; and all of the supernatants were pooled and concentrated using a PM-10 Amicon membrane in an Amicon Model 52 ultrafiltration cell. The concentrate was applied to a QAE-Sephadex A-25 column (3 × 30 cm) which had been equilibrated with 20 mM Tris-HCl buffer, pH 8. After the application of the protein the column was washed with the same buffer and the fractions (30 ml/h) containing *p*-nitrophenyl butyrate (PNB)[3] and cutin hydrolyzing activity were collected. These cutinase-containing fractions were pooled and concentrated by ultrafiltration.

After dialysis against 100 mM Tris-HCl buffer, pH 8, the enzyme solution was applied to a Sepharose 6B column (2 × 100 cm) which was previously equilibrated with 100 mM Tris-HCl, pH 8, and the proteins were eluted with the same buffer at 24 ml/h, collecting 6 ml fractions. The column effluent fractions containing cutinase activity were pooled and concentrated by ultrafiltration. This enzyme preparation was subjected to gel filtration on a Sephadex G-100 column under the same conditions used for the Sepharose 6B column.

Enzyme assays. Hydrolysis of *p*-nitrophenyl esters of fatty acids was measured spectrophotometrically at 405 nm as described earlier (7) after optimizing assay conditions for the present enzyme. The reaction was run in 3 ml of 0.1 M Tris-HCl, pH 9.0, containing 1.6 mM PNB at 23°C for 3 to 4 min during which time linear increases in absorbance were observed. This initial velocity was used for all activity measurements. Cutin hydrolase activity was assayed by incubating appropriate amounts of the enzyme in 1 ml of 0.1 M sodium phosphate buffer, pH 8, containing 4 mg tritiated cutin at 30°C for 20 min. The reaction was stopped by chilling the tubes on ice followed by acidification with 0.1 ml of 1 M HCl and filtration of the reaction mixture through a glass wool plug placed in Pasteur pipet. An aliquot of the filtrate was assayed for radioactivity by liquid scintillation spectrometry. The purified enzyme (10 μg) was incubated in 0.1 M sodium phosphate buffer, pH 8, containing 0.4% Triton X-100 and inhibitors or chelators for 30 min at room temperature prior to cutinase assay. Protein concentration was routinely determined by the method of Lowry *et al.* (9).

Electrophoresis. Cationic polyacrylamide gel electrophoresis was performed according to Ornstein and Davis (10) with 7.5% polyacrylamide resolving gel (7.0 cm), pH 8.0, and 2.5% stacking gel. In order to locate the position of cutinase activity, the gel was sliced into 2-mm slices and soaked overnight in the

[3] Abbreviations used: PNB, *p*-nitrophenyl butyrate; SDS, sodium dodecyl sulfate.

appropriate enzyme assay buffer. Aliquots were assayed for enzyme activity with both ^{3}H-labeled apple cutin and PNB as substrates.

SDS-polyacrylamide gel electrophoresis was performed as described before (11) with 12% polyacrylamide resolving gel, pH 8.8, and 3% stacking gel, pH 6.7, each containing 1% sodium dodecyl sulfate. Protein samples were placed in boiling water for 1 min followed by addition of SDS (1% final concentration) and 2-mercaptoethanol (0.1 M final concentration) and the mixture was held at the same temperature for another 2 min. Slab gel electrophoresis was performed at 25 mA and the gels were stained with Coomassie blue and destained. The following standard proteins were similarly treated before electrophoresis: phosphorylase *b*(92.5 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Estimation of molecular weight of the native enzyme. Gel filtration was performed on a column (1.4 × 105 cm) of Sephadex G-100 equilibrated with 0.1 M sodium phosphate buffer, pH 8, with the same proteins as above as molecular weight standards.

Amino acid analysis. Amino acid analysis was done with a Beckman Model 121 MB automatic amino acid analyzer. Cysteine and methionine were determined after performic acid oxidation (12). Tryptophan was determined by a spectrophotometric method (13).

Hydrolysis of triglycerides. The reaction mixture containing 0.1 M sodium phosphate buffer, pH 8.0, containing 2 nmol tri[1-^{14}C]palmitoyl or tri[1-^{14}C]-oleoylglycerol and 10 μg of enzyme was incubated at 30°C for 3 h with shaking. The reaction mixture was acidified with HCl and the products extracted with chloroform were mixed with authentic palmitic acid and subjected to thin-layer chromatography with hexane:ethyl ether:formic acid (80:20:1) as the developing solvent. After spraying the plate with an ethanolic 0.1% solution of 2,7-dichlorofluoresceine the free fatty acid was located under uv light and the silica gel scraped from the region was assayed for ^{14}C by liquid scintillation spectrometry.

Immunological studies. About 400 μg purified cutinase in 1 ml 10 mM sodium phosphate, pH 7.8, containing 0.9% NaCl, was mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit MI). The mixture was emulsified by sonication and injected into rabbit dorsal subcutaneous sites. Two additional injections of 200 μg of cutinase mixed with incomplete Freund's adjuvant were given at 2-week intervals prior to bleeding. The serum collected 7 days after the second injection was treated with one-half the volume of a saturated ammonium sulfate solution. The resulting immunoglobulin precipitate was collected by centrifugation and dissolved in the original serum volume with 10 mM sodium phosphate-saline buffer, pH 7.8, and dialyzed overnight against the same buffer. The ammonium sulfate fractionation was repeated and the resulting immunoglobulin precipiate was dissolved in one-half of its serum volume and dialyzed overnight against 10 mM sodium phosphate-saline buffer, pH 7.8. The immunodiffusion technique of Ouchterlony (14) was performed in petri dishes containing 2% noble agar (Difco Laboratories) in 100 mM veronal buffer, pH 8.5, containing 0.9% NaCl and 0.01% thimerosal. After diffusion of antigens and antibodies for 16–24 h, the plates were soaked in 0.9% NaCl for 4 days and the immunoprecipitant bands were fixed with 7.5% acetic acid.

Western blots were done using a Hoeffer Transblot apparatus and the buffer of Towbin (15). Cutinase (0.5 to 1.2 μg) was mixed with varying amounts (0 to 1.0 mg) of preimmune or immune rabbit IgGs in 150 μl of 100 mM sodium phosphate buffer, pH 7.6, and bovine serum albumin was added in quantities necessary to maintain a constant protein concentration. After the mixture was incubated at 25°C for 1.5 h, it was left at 4°C overnight before assaying for enzymatic activity by the radioassay with tritiated cutin or by the spectrophotometric assay with PNB as the substrate.

RESULTS AND DISCUSSION

Purification of cutinase. Since the extracellular fluid was intensely colored and viscous but was low in protein concentration, a preliminary acetone fractionation was done to obtain an enriched enzyme preparation. Excellent recovery (80%) of the cutinase activity was noted when the acetone content was 75%. Some of the pigments were removed by acetone fractionation. Most of the remaining pigments were retained by DEAE-52 while cutinase was not. The remaining coloration associated with the enzyme preparation was removed by a QAE-Sephadex column. A major loss of PNB hydrolyzing activity was seen at this stage, perhaps because this step removed a PNB hydrolase other than the cutinase that might have been present in the extracellular fluid. Up to this stage, an 80-fold purification was achieved with a recovery of about 9% of enzyme activity (Table I). All attempts to further purify the enzyme by cation and hydrophobic interaction chromatography with SP-Sephadex, CM-cellulose, phenyl-Sepharose, and octyl-Sepharose failed because cutinase did not bind to these gels.

TABLE I

SUMMARY OF PURIFICATION OF CUTINASE FROM FLUORESCENT *Pseudomonas putida* BACTERIAL STRAIN

Step	Protein (mg)	Activity (total units)	Specific activity (μmol/min/mg)	% Recovery	Fold purification
Extracellular fluid	7360	249,450	44	100	1
Acetone precipitation	2090	182,000	87	78	3
DEAE-52	460	121,200	262	50	8
QAE-Sephadex	11	22,000	2650	9	78
Sepharose 6B	4	14,500	3360	6	99
Sepharose G-100	1.1	4,430	7030	3	207

Upon gel filtration on a Sepharose 6B column (Fig. 1, top) a 100-fold purification was achieved and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the resulting enzyme preparation showed only two bands (Fig. 2). The contaminant protein was removed by gel filtration on a Sephadex G-100 column (Fig. 1, bottom). By the combination of the five steps about

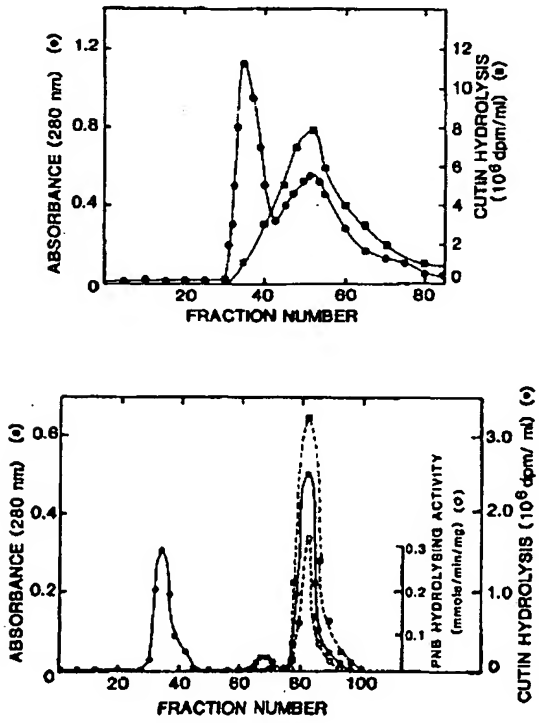


FIG. 1. Top, Sepharose 6B gel filtration of the *Ps. putida* cutinase preparation obtained from a QAE-Sephadex column. Botton, Sephadex G-100 gel filtration of the cutinase preparation obtained from the Sepharose 6B step shown on top. Experimental details are described in the text.

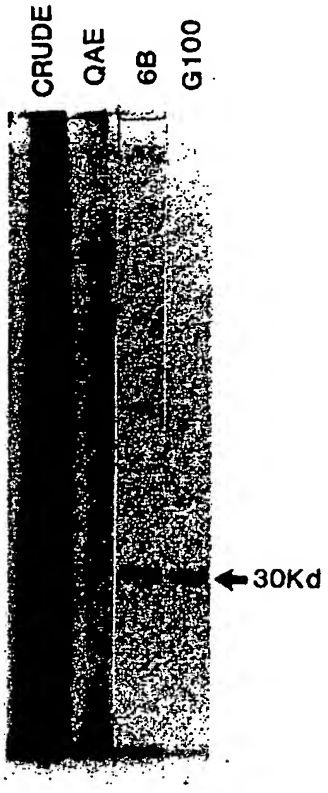


FIG. 2. SDS-gel electrophoresis of the *Ps. putida* cutinase preparations at different stages of purification. Crude extracellular fluid, QAE-Sephadex, Sepharose 6B, and Sephadex G-100 column steps are indicated. Electrophoresis was carried out with molecular weight standards as described in the text.

ERIAL STRAIN

Fold purification
1
3
8
78
99
207

ltration on a
g. 1, bottom).
/e steps about

30Kd

f the *Ps. putida*
ages of purifica-
AE-Sephadex,
olumn steps are
ed out with mo-
d in the text.

200-fold purification was achieved with a recovery of about 3% enzyme activity (Table I). The major loss in enzyme activity resulted from the QAE-Sephadex step. Elution of the QAE column with detergents resulted in the elution of additional amounts of enzyme activity. However, this preparation contained brown color which could not be removed by subsequent steps. Therefore, yield was sacrificed for purity. Cutinase prepared as above was pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as shown in Fig. 2. Only a 200-fold purification was necessary to attain homogeneity because of the relatively high abundance of the enzyme in cell-free extract as revealed by dodecyl sulfate electrophoresis of the total extract (Fig. 2).

Evidence for homogeneity. Sephadex G-100 filtration showed a single symmetrical peak of protein which was coincident with the enzyme activity profile. Polyacrylamide gel electrophoresis showed only one protein band and all of the enzymatic activity, as measured with PNB and labeled cutin as substrates, corresponded to this protein band (Fig. 3).

Molecular properties of bacterial cutinase. The molecular weight of the monomer was estimated by dodecyl sulfate-electrophoresis to be 30,000 (Fig. 2). The native molecular weight of 30,000 was estimated by gel filtration through a calibrated Sephadex G-100 column (data not shown). Therefore, it is concluded that native enzyme is a monomer of molecular weight 30,000.

The amino acid composition of bacterial cutinase is different from that of fungal or plant cutinase (Table II). The substantial differences are seen in the case of methionine, histidine, and lysine residues. For example, only one or two histidine residues are present in fungal cutinases whereas five histidine residues are present in the bacterial and plant cutinases.

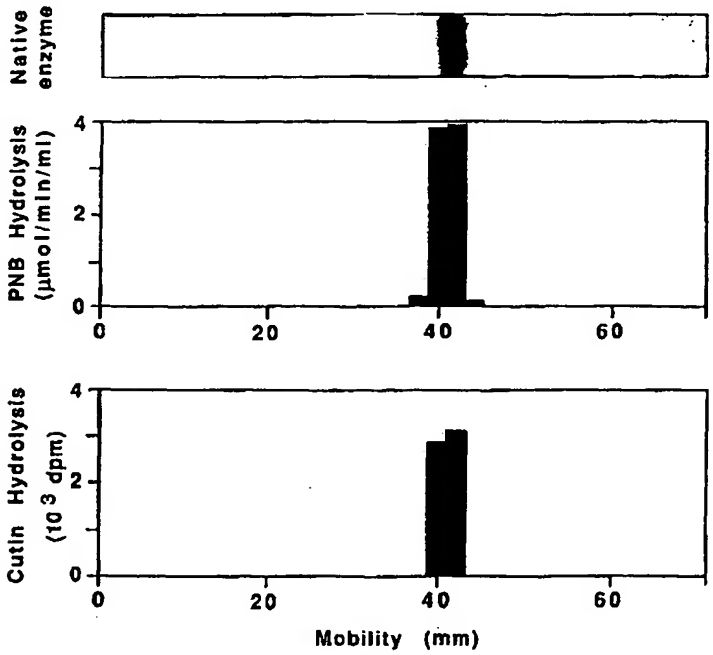


FIG. 3. Polyacrylamide disk gel electrophoresis of cutinase obtained from the Sepharose G-100 step shown in Fig. 1. One gel was stained with Coomassie blue (top) and the other was sliced and assayed for enzyme activity as described under Materials and Methods.

TABLE II

AMINO ACID COMPOSITION OF *Pseudomonas* CUTINASE COMPARED TO POLLEN AND FUNGAL CUTINASES

Amino acid	Number of residues per molecule		
	Fungi[a]	*Nasturtium*[b] pollen	*Pseudomonas*
Asx	22	48	27
Thr	14	22	16
Ser	17	20	18
Glx	16	48	29
Pro	11	11	18
Gly	28	26	34
Ala	28	29	28
Cys	4	7	4
Val	11	24	15
Met	1	4	4
Ile	13	17	9
Leu	21	24	24
Tyr	6	6	9
Phe	7	17	11
Lys	8	20	1
His	2	5	5
Arg	13	9	16
Trp	1	N.D.	1

[a,b] Values from Refs. (6) and (18), respectively.

Immunological properties. Rabbit antiserum perpared against bacterial cutinase showed a single sharp precipitin line when tested against the antigen by the Ouchterlony double diffusion technique (Fig. 4A). Western blot analysis of the total extracellular proteins produced by induced *Ps. putida* gave a single band (Figure 4B) corresponding to a 30 kDa protein, suggesting that the antibody is specific for cutinase. With increasing concentrations of the antibody (Fig. 4C) almost complete inhibition of the enzymatic activity was observed. Cutin degrading activity showed a higher degree of sensitivity than PNB hydrolyzing activity possibly because the accessibility of the large insoluble cutin polymer to the active site of the enzyme is more readily affected than the accessibility of the small model ester by the binding of the antibody. Rabbit antiserum prepared against cutinase I from *Fusarium solani pisi* did not cross-react with the bacterial enzyme and the activity of fungal enzyme was not affected by the antiserum prepared against the bacterial enzyme (data not shown).

Effects of pH, time, protein concentration, and cutin concentration on cutin hydrolysis. The bacterial cutinase was characterized using tritiated apple cutin as the substrate and the assay involved the enzymatic release of soluble cutin components from the insoluble labeled cutin. Below pH 7, the rate of cutin hydrolysis was very low, but the rate increased rapidly as the pH was raised from 7.5 to 10.0 showing a broad pH optimum between 8.5 to 10.5 (Fig. 5). With increasing amounts of cutin, the rate of hydrolysis increased linearly up to 4 mg/ml of cutin and further increase in the substrate content of the reaction mixture did not change the rate. The slight decrease in the hydrolysis rate might be due to the absorption of the enzyme on the insoluble cutin polymer. Rectilinear rates of cutin hydrolysis were observed up to 20 min of incubation time. With increasing enzyme concentration there was a rectilinear increase in the rate of hydrolysis.

Substrate specificity. The bacterial cutinase catalyzed hydrolysis of *p*-nitrophenyl esters of C_4 to C_{16} fatty acids. With all these substrates typical Michaelis-Menten type kinetics were observed. From linear double-reciprocal plots K_m values and V were calculated (Table III). There was a general trend of increasing K_m and decreasing V as the chain length of acyl group was increased. However, these changes were not as large as those observed with some fungal cutinases. With many of the fungal enzymes, as the chain length of the acyl moiety increased from C_2 to C_{12} V decreased 200- to 1000-fold.

Effect of inhibitors. Thiol-directed reagents such as *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, and iodoacetamide at 2 and 5 mM had no effect on cutinase (data not shown). Active serine-directed reagents such as diisopropylfluorophosphate at 25 μM and (0,0)-diethyl-(3,5,6-trichloro)-2-pyridylphosphorothioate at 1 mM inhibited cutinase activity by 90%. Phenylboronic acid, a

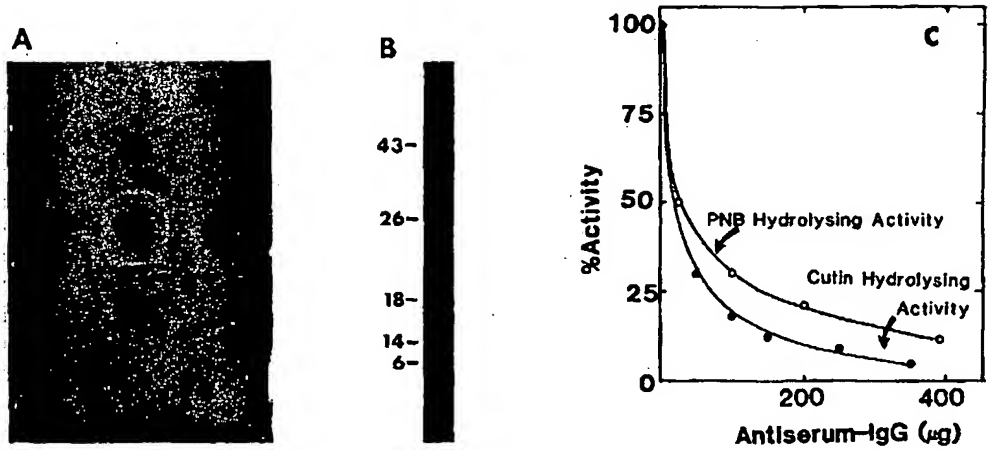


FIG. 4. (A) Ouchterlony double diffusion analysis using rabbit anticutinase at the center and four different concentrations of purified bacterial cutinase (200, 400, 600, 800 ng) in the four wells. (B) Autoradiogram of a Western blot of the extracellular fluid of induced *Ps. putida* probed with antibody raised against purified cutinase. Lyophilized extracellular fluid (0.5 ml) was dialyzed and the protein precipitated by trichloroacetic acid was run on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose sheet which was incubated with anticutinase IgG followed by treatment with ^{125}I-labeled protein A. (C) Inhibition of *Ps. putida* cutinase by rabbit anticutinase IgG. Increasing amounts of anticutinase IgG were preinubated with the enzyme and the residual enzyme activities were determined by either the tritiated cutin hydrolysis assay or the spectrophotometric assay with PNB as described in the text.

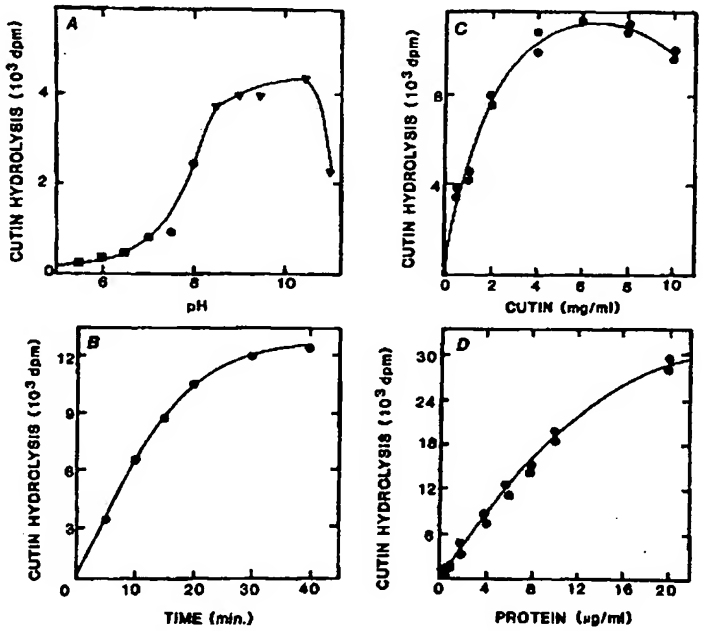


FIG. 5. Effects of pH (A), incubation time (B), amount of the substrate (C), and protein concentration (D) on the hydrolysis of apple cutin by purified *Ps. putida* cutinase. Except for the variable factor, the assay conditions included 0.6 μg of cutinase and 4.0 mg of [^{3}H]cutin in 1 ml Tris-HCl buffer, pH 9, incubated for 20 min at 30°C. The reaction mixtures for the pH curve (A) contained citrate phosphate (■), Tris-HCl (●), or glycine-NaOH (▲) buffers.

TABLE III

EFFECT OF THE CHAIN LENGTH OF THE ACYL MOIETY OF *p*-NITROPHENYL ESTERS ON K_m AND V VALUES FOR HYDROLYSIS BY THE *Ps. putida* CUTINASE

Chain length of acyl moiety	V^a	K_m (M × 10^{-4})
C_4	77	2.7
C_6	18	2.1
C_8	35	17.6
C_{10}	16	39.8
C_{12}	14	35.5
C_{14}	8	22.7
C_{16}	9	45.4

[a] V is expressed as μmol/min/ml of protein.

transition state analog of serine hydrolases (8,16,17), inhibited cutinase activity by 63 and 80% at 5 and 10 mM respectively. The inhibition of cutinase by phenylboronic acid was competitive in nature (Fig. 6). From these results, it is concluded that the present bacterial cutinase uses a catalytic triad involving active serine for catalysis like the fungal enzymes. Thus, it differs from the pollen enzyme which was suggested to have an SH group at the active site (18). Cutinase activity was not affected either by chelators such as EDTA and 8-hydroxyquinoline or by metal ions such as Ca^{2+}, Mg^{2+}, and Cu^{2+}. Alkylboronic acids with different chain lengths were synthesized and K_i values were determined using different alkylboronic acids (Table IV). Phenylboronic acid was less inhibitory compared to alkylboronic acids, and as the chain length decreased the K_i values decreased, suggesting that the binding site does not have a large hydrophobic pocket.

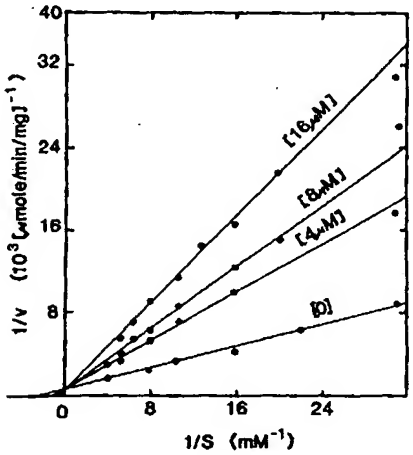


FIG. 6. Inhibition of cutinase by phenylboronic acid. The reaction mixture contained the indicated concentrations of the inhibitor.

The present results reveal that the bacterial enzyme is quite different from fungal and plant cutinases. The molecular weight of bacterial enzyme (30,000) is smaller than that of pollen enzyme (40,000) and larger than those of the fungal enzymes (25,000) so far examined. The amino acid composition of the present enzyme is quite different from those of the pollen and fungal enzymes. Major differences are seen in the case of histidine, lysine, and methionine residues. The K_m and V values, calculated for model substrates, reveal that bacterial cutinase is similar to pollen enzyme but different from fungal enzymes. The substrate specificity and pH optima of bacterial and fungal enzymes are comparable. The catalytic mechanism used by the bacterial enzyme resembles that of fungal cutinases in that both use the active serine catalytic triad. In this respect, it differs from pollen enzyme which has a thiol group at the active site.

The function of the present bacterial cutinase is quite different from those of fungal and plant cutinases previously reported (18-20). The pollen cutinase might be involved in the penetration of stigma cuticle by the pollen tube and thus might play a role in self-incompatability in fer-

TABLE IV

CUTINASE INHIBITION BY BORONIC ACIDS

Side chain	Value K_i (μM)
Phenyl	115
C_{16}	19.9
C_{14}	14.4
C_{12}	10.7
C_{10}	6.9
C_8	4.9
C_4	3.2

tilization. Fungal phytopathogens use cutinase to penetrate the cuticular barriers during infection (21). The only previously studied bacterial cutinase was that produced by *Streptomyces scabies* (20). Since it has been shown that cutinase hydrolyzes the polyester domains of suberin (22) it seems likely that the major function of the enzyme from *S. scabies* is to break down the suberized periderm layer that protects potato tuber, a natural host of this pathogen. The present bacterial enzyme might play a novel role of providing nutrients for a nitrogen-fixing *Corynebacterium* sp. that cohabits with *Ps. putida* in the phyllosphere (5). Cutinase-producing *Ps. putida* was coisolated with a *Corynebacterium* sp. and our studies suggest that *Corynebacterium* sp. can provide reduced nitrogen required for the growth of *Ps. putida* strain and the latter can provide a carbon source for the growth of the former (5). Thus, both can survive together with the plant cuticular polymer as the sole source of carbon without any exogenous source for reduced nitrogen. An additional feature of the phyllospheric system is that the *Corynebacterium* sp. presumably provides nitrogen to the host plant as well as the associative microbial partner. The demonstration that the combined bacterial culture sprayed on the plants can significantly increase yield of wheat which received no nitrogen fertilizer (4) suggests that the contribution of reduced nitrogen from the phyllospheric bacteria might be significant under the tropical conditions. The purification and characterization of the cutinase could help us to perform molecular level tests to determine the precise role of cutinase in this mutually beneficial association between these two bacteria and the plant.

REFERENCES

1. RUINEN, J. (1974) *in* The Biology of Nitrogen Fixation (Quespel, A., Ed.) pp. 121-169, Elsevier/North-Holland, Amsterdam.
2. VASANTHARAJAN, V. N. AND BHAT, J. V. (1968) *Plant Soil* 28, 258-267.
3. LADHA, J. K., BARRAQUIO, W. L., AND WATANABE, I. (1983) *Canad. J. Microbiol.* 29, 1301-1308.
4. PATTI, B. R., AND CHANDRA, A. K. (1981) *Plant Soil* 61, 419-427.
5. SEBASTIAN, J., CHANDRA, A. K., AND KOLATTUKUDY, P. E. (1987) *J. Bacteriol.* 169, 131-136.
6. KOLATTUKUDY, P. E. (1982) *in* Lipases (Borgstrom, B., and Brockman, H. Eds.), pp. 471-504, Elsevier/North-Holland, Amsterdam.
7. KOLATTUKUDY, P. E., PURDY, R. E., AND MAITI, I. B. (1981) *in* Methods in Enzymology (Lowenstein, J. M., Ed.), Vol. 71, pp. 652-654, Academic Press, Orlando, FL.
8. KRAUT, J. (1977) *Annu. Rev. Biochem.* 46, 331-358.
9. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* 193, 265-275.
10. ORNSTEIN, L., AND DAVIS, B. (1964) *Ann. N.Y. Acad. Sci.* 121, 404-408.
11. WEBER, K., PRINGLE, J. R., AND OSBORN, M. (1972) *in* Methods in Enzymology (Hirs, C. H. W., and Timasheff, S. N., Eds.), Vol. 26, Part C, pp. 3-27, Academic Press, New York.
12. HIRS, C. H. W. (1967) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., Eds.), Vol. 2, pp. 59-62, Academic Press, New York.
13. MATSUBARA, H., AND SUSAKI, R. M. (1969) *Biochem. Biophys. Res. Commun.* 35, 175-181.
14. OUCHTERLONY, O. (1953) *Acta Pathol. Microbiol. Scand.* 32, 231-240.
15. TOWBIN, H. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350-4354.
16. KOEHLER, K. A., AND HESS, G. P. (1974) *Biochemistry* 13, 5345-5350.
17. GARNER, C. W. (1980) *J. Biol. Chem.* 255, 5064-5068.
18. MAITI, I. B., KOLATTUKUDY, P. E., AND SHAYKH, M. (1979) *Arch. Biochem. Biophys.* 196, 412-423.
19. PURDY, R. E., AND KOLATTUKUDY, P. E. (1975) *Biochemistry* 14, 2832-2840.
20. LIN, T. S., AND KOLATTUKUDY, P. E. (1980) *Physiol. Plant. Pathol.* 17, 1-15.
21. KOLATTUKUDY, P. E. (1985) *Annu. Rev. Phytopathol.* 23, 223-250.
22. FERNANDO, G., ZIMMERMANN, W., AND KOLATTUKUDY, P. E. (1984) *Physiol. Plant. Pathol.* 24, 143-155.

Comparison of:
(A) ./wwwtmp/lalign/.26656.1.seq Pseudomonas mendocina
- 227 aa
(B) ./wwwtmp/lalign/.26656.2.seq Thermobifida fusca
- 261 aa
using matrix file: BL50, gap penalties: -14/-4

26.0% identity in 131 aa overlap; score: 132 E(10,000): 3.5e-05

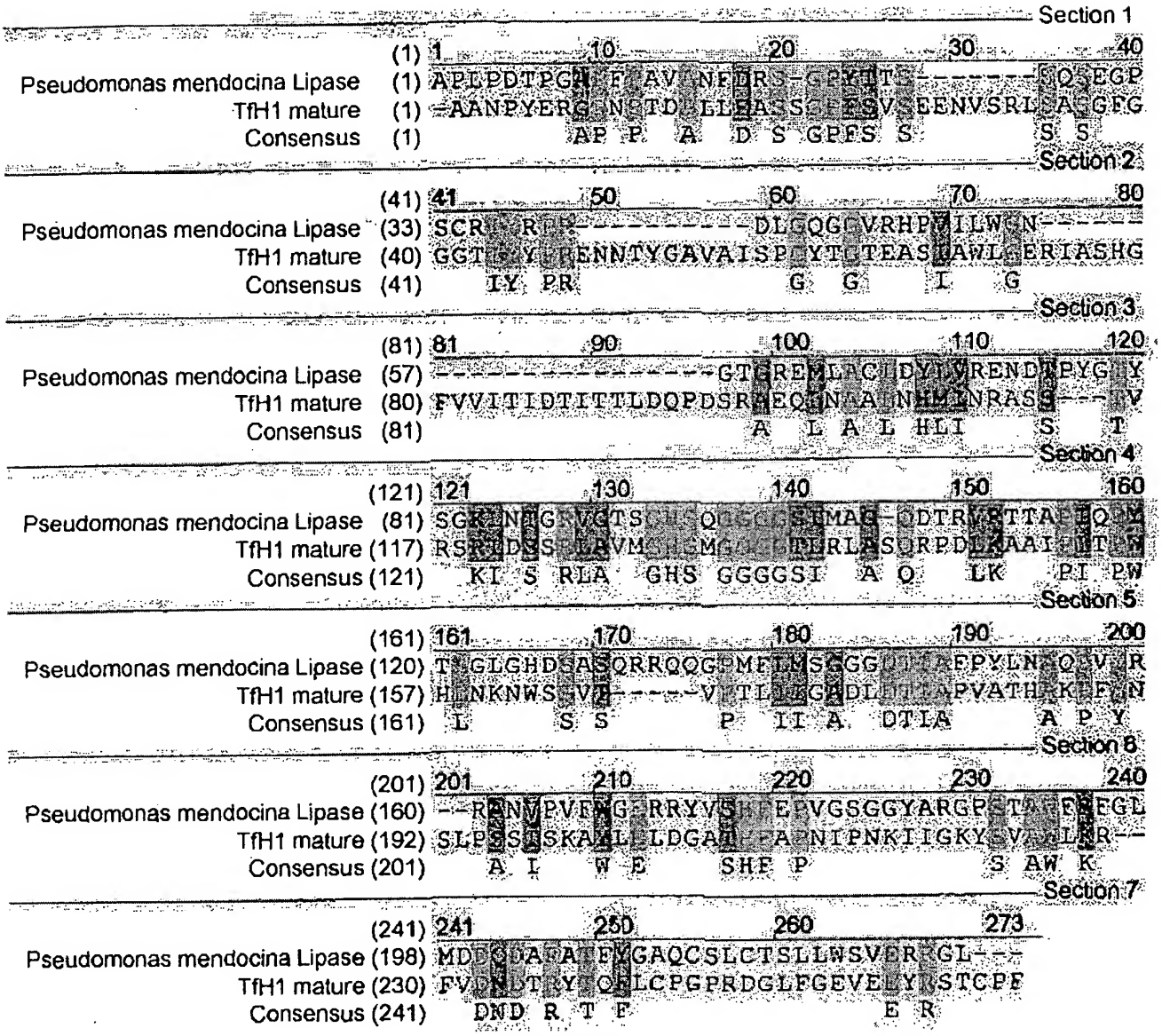
```
       80        90       100       110       120       130
Pseudo TYSGKLNTGRVGTSGHSQGGGGSI-MAGQDTRVRTTAPIQPYTLGLGHDSASQRRQQGPM
       :  .....:... :::.:::::.. .:.:   .... :. :. :. . .:..       :
Thermo TVRSRIDSSRLAVMGHSMGGGGTLRLASQRPDLKAAIPLTPWHLNKNWSSVTV-----PT
           120       130       140       150       160

       140       150       160       170       180       190
Pseudo FLMSGGGDTIAFPYLNAQPVYRR--ANVPVFWGERRYVSHFEPVGSGGYARGPSTAWFRF
       .....  ::::    .:.: :    ...   . :   ..:: :   .       :.::..
Thermo LIIGADLDTIAPVATHAKPFYNSLPSSISKAYLELDGATHFAPNIPNKIIGKYSVAWLKR
      170       180       190       200       210       220

         200
Pseudo GLMDDQDARAT
        .  :.:.: :
Thermo FV--DNDTRYT
      230
```

25.0% identity in 56 aa overlap; score: 51 E(10,000): 5.5e+02

```
        70        80        90       100       110       120
Pseudo DYLVRENDTPYGTYSGKLNTGRVGTSGHSQGGGGSIMAGQDTRVRTTAPIQPYTLG
       : :.. .. :...  .. :..:...::    :::.:.  ... .  .. :.:   :
Thermo DALLEASSGPFSV--SEENVSRLSASGF---GGGTIYYPRENNTYGAVAISPGYTG
             20          30          40        50        60
```

54.5% identity in 11 aa overlap; score: 40 E(10,000): 4.1e+03

```
           150
Pseudo GGDTIAFPYLN
       :: :: .:  :
Thermo GGGTIYYPREN
        40
```


Section 1
Section 2
Section 3
Section 4
Section 5
Section 6
Section 7
Pseudomonas mendocina Lipase
TfH1 mature
Consensus